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ORIGINAL ARTICLE

Allergens

Influence of peanut matrix on stability of allergens in gastric-simulated digesta: 2S albumins are main contributors to the IgE reactivity of short digestion-resistant peptides

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Summary

Background: Most food allergens sensitizing via the gastrointestinal tract are stable proteins that are resistant to pepsin digestion, in particular major peanut allergens, Ara h 2 and Ara h 6. Survival of their large fragments is essential for sensitizing capacity. However, the immunoreactive proteins/peptides to which the immune system of the gastrointestinal tract is exposed during digestion of peanut proteins are unknown. Particularly, the IgE reactivity of short digestion-resistant peptides (SDRPs; <10 kDa) released by gastric digestion under standardized and physiologically relevant in vitro conditions has not been investigated.

Objective: The aim of this study was to investigate and identify digestion products of major peanut allergens and in particular to examine IgE reactivity of SDRPs released by pepsin digestion of whole peanut grains.

Methods: Two-dimensional gel-based proteomics and shotgun peptidomics, immunoblotting with allergen-specific antibodies from peanut-sensitized patients, enzyme-linked immunosorbent inhibition assay and ImmunoCAP tests, including far ultraviolet-circular dichroism spectroscopy were used to identify and characterize peanut digesta.

Results: Ara h 2 and Ara h 6 remained mostly intact, and SDRPs from Ara h 2 were more potent in inhibiting IgE binding than Ara h 1 and Ara 3. Ara h 1 and Ara h 3 exhibited sequential digestion into a series of digestion-resistant peptides with preserved allergenic capacity. A high number of identified SDRPs from Ara h 1, Ara h 2 and Ara h 3 were part of short continuous epitope sequences and possessed substantial allergenic potential.

Conclusion and Clinical Relevance: Peanut grain digestion by oral and gastric phase enzymes generates mixture of products, where the major peanut allergens remain intact and their digested peptides have preserved allergenic capacity highlighting their important roles in allergic reactions to peanut.

Prodic and Stanic-Vucinic are equally contributing authors.

KEYWORDS

digestion-resistant peptides, food matrix, gastric-simulated digestion, peanut allergy, proteolysis resistance

1 | INTRODUCTION

Peanut (*Arachis hypogaea*) is 1 of the 8 major allergenic foods, called the Big-8, which are mandatorily declared for any processed food products according to US and EU regulations. In addition, a low eliciting dose and high frequency of fatal reactions make peanut 1 of the most potent allergenic foods.¹ The major allergens in peanut are Ara h 1, Ara h 2, Ara h 3 and Ara h 6, based on frequent IgE-binding studies from patient sera.² However, based on the potency of the allergic effector activity of the allergens and loss of potency upon their removal, Ara h 2 and Ara h 6 are regarded as the most potent peanut allergens.³

Food ingestion is the major route of sensitization to food allergens. A great number of allergenic proteins are remarkably resistant to proteolysis in the gastrointestinal tract, and survival of their large fragments is essential for their sensitizing capacity.⁴ There is no strict relationship between digestibility and allergenicity, and several major allergens are extremely digestion-labile proteins. However, evaluation of resistance to digestion with pepsin by in vitro simulated digestion remains a central part of allergenicity safety assessment of novel proteins.⁴ In vitro methods simulating digestion processes are widely used tools because of their simplicity, low cost, reproducibility and ethical acceptability.⁵

Several studies have investigated pepsin digestibility of peanut allergens by in vitro simulated digestion (Table S1), and regardless of the experimental conditions, Ara h 2 and Ara 6 showed higher pepsin resistance compared to Ara h 1 and Ara h 3.^{6,7} In most studies, purified peanut allergens or peanut extracts were digested. Only 2 studies have dealt with peanut protein digestibility within their real food matrix. Plundrich et al⁸ investigated digestibility of light-roasted peanut flour (12% fat) by pepsin and monitored degradation of allergens using 1-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE). The study of Di Stasio et al⁹ investigated digestibility of proteins from the whole peanut by complete oral-gastric-intestinal-brush border membrane proteases, but it did not report the gastric digestion products. Therefore, the immunoreactive protein/peptide species to which the immune system of the gastrointestinal tract is exposed during digestion of peanut proteins remains unknown. In particular, the IgE reactivity of short digestion-resistant peptides (SDRPs) released by gastric digestion is yet to be investigated. This fraction also contains important highly immunoreactive peptides. It has been demonstrated that some Ara h 2 peptides of size <3 kDa can cross-link IgE/Fc ϵ RI complexes and degranulate cells,¹⁰ and that Ara h 1, when digested to small peptide fragments, retains both the sensitizing and the IgE-reacting potential because of the peptide's ability to aggregate.¹¹

The aim of this study was to comprehensively investigate stability and structures of pepsin-resistant allergens, of their larger fragments and of SDRPs released by pepsin digestion of whole peanut grain under standardized and physiologically relevant gastric conditions.⁵ In particular, IgE reactivity of SDRPs released by pepsin digestion was investigated to determine roles of individual peanut allergens digestion products.

2 | METHODS

2.1 | Materials

All details and information about materials and chemicals are available in Methods S1.

2.2 | Patients' cohort and ethics statement

Sera from 10 Swedish peanut-sensitized patients with IgE levels to whole peanut extract (range 11–415 kU_A/L; median 57 kU_A/L), Ara h 1 (range <0.1–96 kU_A/L; median 7.8 kU_A/L), Ara h 2 (range 0.14–192 kU_A/L; median 30 kU_A/L) and Ara h 3 (range <0.1–52 kU_A/L; median 1.6 kU_A/L) (Phadia/Thermo Fisher Scientific, Uppsala, Sweden) were selected at the Department of Clinical Immunology, Karolinska University Hospital, Stockholm (Table S3). Five non-allergic sera (<0.1 kU_A/L) were used as controls. Sera were either used individually or pooled. Data were processed and stored according to the principles expressed in the Declaration of Helsinki. The study was approved by the local ethics committee of Karolinska Institute (No. 20112085-314 and 2016/1348-32), and all experiments were in accordance with relevant guidelines and regulations. Sample collection was performed blinded.

2.3 | Peanut preparation, simulated oral and gastric in vitro digestion and protein extract isolation

Red skin raw peanuts (*Arachis hypogaea* L.) of the runner variety were obtained from a local grocery. Raw peanuts with skin were milled using a coffee grinder (800 W; Bosh), 3 times, 5 minutes to obtain a particle size <1.5 mm. Ground peanuts were air-dried overnight at room temperature (the amount of evaporated water was 5.4% of total mass).

Oral and gastric in vitro digestions of ground raw peanut were performed according to the previously reported method.⁵ The concentrations of electrolytes in stock solutions of simulated salivary fluid (SSF), simulated gastric fluid (SGF) and the final reaction

mixtures are presented in Table S2 (more details are available in Supporting Information).

The liquid phase of the digestion mixture (200 μ L) was mixed with 200 μ L chilled 20% trichloroacetic acid (TCA) in acetone and left overnight at -20°C . After removing the supernatant by centrifugation at 10 000 g for 30 minutes at 4°C , the precipitated proteins were washed 3 times with 1 mL of cold acetone and dried at RT. Protein concentration was determined using BCA assay after re-solubilization of TCA/acetone pellet in 2% SDS. On the other hand, 800 μ L of the liquid phase of the digestion was processed without TCA to obtain SDRPs, as described below.

2.4 | 1D and 2D SDS-PAGE analysis

1D SDS-PAGE was performed on a 14% gels according to Laemmli method¹² and stained with Coomassie Brilliant Blue dye. Dried TCA/acetone protein pellets from liquid portion of gastric-simulated digesta were re-suspended in Laemmli sample buffer (reducing and non-reducing conditions). Isoelectrofocusing and 2D SDS-PAGE were performed as per the method of Apostolovic et al.¹³ For more details, see Supporting Information.

2.5 | In-gel digestion of 2D SDS-PAGE proteins and nano-liquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS)

Protein spots from 2D gels were manually excised and in-gel digested, according to the method of Shevchenko et al.¹⁴ Obtained peptides were analysed, as previously reported method,^{13,15} with an LTQ Orbitrap XL mass spectrometer and EASY-nLC II system (Thermo Fisher Scientific Inc., Bremen, Germany).

binding tubes. The dried peptides were dissolved in 10 mmol/L HCl and subjected to size-exclusion chromatography (Figure S1). A portion of peptides was analysed by ImmunoCAP inhibition assay. The second part was subjected to shotgun peptidomics analyses as explained in Supporting Information. Peptides were searched in the IEDB database (Immuno Epitope Database and Analysis, <http://www.iedb.org>) to find sequences overlapping with characterized epitopes.

2.8 | IgE-binding properties of peanut digests

2.8.1 | ELISA inhibition

The IgE-binding properties of the liquid phase from the digestion mixtures (control peanut sample [CPS] and digested peanut sample [DPS]), as well as standard defatted peanut extracts, were analysed using an inhibition ELISA. Standard defatted raw peanut extract was prepared according to the method reported by Radosavljevic et al.¹⁶ with all patients (Table S3). For more details, see Supporting Information.

2.8.2 | ImmunoCAP inhibition

IgE binding of the SDRPs fraction of digested peanut was determined using ImmunoCAP inhibition (ImmunoCAP System; Phadia/Thermo Fisher Scientific). Seven undiluted individual sera (200 μ L; patients #1-7 Table S2) were pre-incubated with 200 μ L peptides prior to the measurement for specific IgE on solid surface for the following: peanut (f13), Ara h 1 (f422), Ara h 2 (f423) and Ara h 3 (f424). Applied peptides were released from about 3.3 mg of milled peanut, for example released from about 800 μ g of peanut proteins extracted to liquid phase during digestion. The inhibition of IgE binding was expressed as percentage based on non-inhibited serum, using the following formula:

$$\% \text{ IgE inhibition} = 100 - \frac{\text{IgE binding to the solid surface in the presence of the inhibitor}}{\text{IgE binding to the solid surface}} \times 100.$$

2.6 | Identification of standard extract, control and digested peanut proteins from 2D SDS-PAGE

Identification of proteins was performed by PEAKS Studio 8.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada). For more details, see Supporting Information.

2.7 | Separation of SDRPs obtained after gastric-simulated digestion and their identification via shotgun peptidomics

Ethanol (2.4 mL) was added to 800 μ L of liquid phase separated from the digestion mixture and incubated at 4°C for 20 hours. After centrifugation at 4°C and 12 000 g for 10 minutes, the supernatant with SDRPs was separated and dried in a vacuum concentrator in low

2.8.3 | Immunoblotting

The samples (120 μ g for 2D immunoblots on 7 cm IPG strips) were loaded and resolved on 14% gel. Proteins were transferred onto nitrocellulose membranes and incubated overnight at 4°C with 1:10 diluted serum pool from patients sensitized to peanut. The serum pool consisted of sera of the first 8 peanut-sensitized patients presented in Table S3 (range of total peanut-specific IgE: 11-415 kU_A/L; median 109 kU_A/L). For more details, see Supporting Information.

2.9 | De novo modelling and molecular graphics

The sequences of Ara h 1 and Ara h 3 were obtained from UniProt (<http://www.uniprot.org>, identifiers P43238 and B5TYU1,

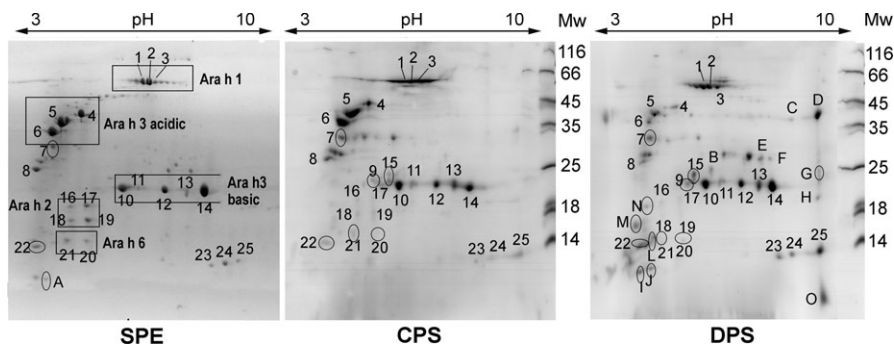


FIGURE 1 2D SDS-PAGE of standard peanut extract (SPE), control peanut sample (CPS) and digested peanut sample (DPS) in reducing conditions. Protein spots labelled with numbers are matched, while non-matched spots are labelled with capitalized letters; all of them were trypsin digested and subjected to MS/MS analyses for identification. Mw, molecular weight protein markers in kDa

respectively). The missing regions in the Ara h 1 and Ara h 3 partial crystal structures (PDB code 3SMH and 3C3V, respectively)¹⁷ were built using Rosetta all-atom de novo loop modelling. For more details, see Supporting Information.

3 | RESULTS

3.1 | Comparison of 2D SDS-PAGE resolved proteomes of standard peanut extract (SPE), CPS and DPS

Whole peanut grains were digested in simulated oral and gastric fluid using standardized in vitro static digestion protocol mimicking human physiological conditions. About 30% of proteins were extracted into the liquid phase during applied simulated oral-gastric digestion (data not shown). Due to the dependence of protein release on food matrix effects,¹⁸ we analysed DPS, SPE prepared from non-digested, defatted peanuts and the CPS treated under the same conditions of digestion (without addition of proteolytic enzymes). In all of them, proteins were TCA precipitated from liquid portion of extract or gastric digesta for 2D SDS-PAGE and 2D immunoblots analyses.

To gain better insight into digestibility pattern of peanut allergens, 2D SDS-PAGE proteome maps of SPE, CPS and DPS were compared (Figure 1). In addition, 1D electrophoretic profiles of CPS and DPS (Figure S2) are explained in detail within Supporting Information. The identified proteins and peptides from spots and bands are listed in Table S4 and correspond to annotated spot maps, as shown in Figures 1 and 3. The 2D electrophoretic profile of CPS was similar to the profile of SPE. The major difference in 2D maps between CPS and DPS is in quantity of acidic subunits of Ara h 3, being substantially less in DPS (spots 4-6 Figure 1), probably due to pepsin proteolysis. In addition, there is a substantial accumulation of protein fragments lower than 14 kDa in acidic region of DPS gel (spots 22, I, J and unlabelled). Spots in the basic part of the gel, D, G, H and O, representing digestion fragments are exclusively present in DPS (Figure 1, Table S4).

3.2 | Ara h 1 and Ara h 3 allergens' cascade pattern of pepsin proteolysis

It was possible to draw pepsin proteolytic pattern for Ara h 1 and Ara h 3 due to its high content within peanut proteome, by

determining the peptides sequence coverage for each of their isoforms within spots (Table S4, Figure 2A,B). Intact Ara h 1 and 3 content at 65 kDa is similar between 3 preparations; however, in DPS, set of their isoforms is positioned at lower mass, approximately at 60 kDa (spots 1-3, Figure 1), implying that both N-terminal and C-terminal non-core flanking regions of Ara h 1 are highly prone to proteolysis (Figure 2A). This 60 kDa fragment (almost intact molecule) is further truncated by pepsin at the C-terminal region, resulting in an intense spot D and pale spot C at about 40 kDa in DPS (Figures 1 and 2A). Further truncation at C-terminal in the middle of the first loop generated fragments (Figure 2A) observed in spot G (Figure 1), while its additional cleavage at N-terminal and C-terminal region resulted in spot H (Figure 2A). Spots M and L in DPS (Figure 1) contain peptides from the C-terminal region of Ara h 1.

Intact acidic Ara h 3 subunit was identified in spot 5 of DPS and compared to the CPS spots which showed a remarkably lower intensity suggesting intensive proteolysis by pepsin. Spots E and F appearing only in DPS contained peptides from acidic and basic Ara h 3 subunits (Figures 1 and 2B). In the spots 9-15, intact or only slightly shortened basic subunit of Ara h 3 in different isoforms was found, with intensities similar to that of the control, suggesting that the basic Ara h 3 subunit is more resistant to pepsin. In the spots N and M (exclusive to DPS, Figure 1), peptides from basic Ara h 3 subunit were detected, being acidic due to N-terminal and C-terminal proteolysis of fragments rich in basic residues. On the contrary, spots in the basic region (23-25, Figure 1) that were the most intense in DPS contained basic peptides from acidic Ara h 3 subunit, due to lack of its acidic residues at C-terminus (Figure 2B). Beside 2S albumins, in CPS we detected Ara h 8 in spot 20 and Ara h 10 in spot 18 (Figure 1, Table S4), but not in 2D gel of DPS.

3.3 | Ara h 2 and Ara h 6 remained almost intact during pepsin digestion

Intact Ara h 2 and 6 were identified in all the peanut proteome preparations, SPE, CPS and DPS (Figures 1 and 3, Table S4). Mass spectrometry identification of Ara h 2 and Ara h 6 spots was acquired from the CPS and DPS gels as shown in Figure 3.

Spot's intensity of Ara h 2 and 6 in CPS and DPS was lower compared to SPE (Figure 1), presumably due to less favourable extraction conditions for 2S albumins in acidic conditions (CPS and DPS).¹⁹

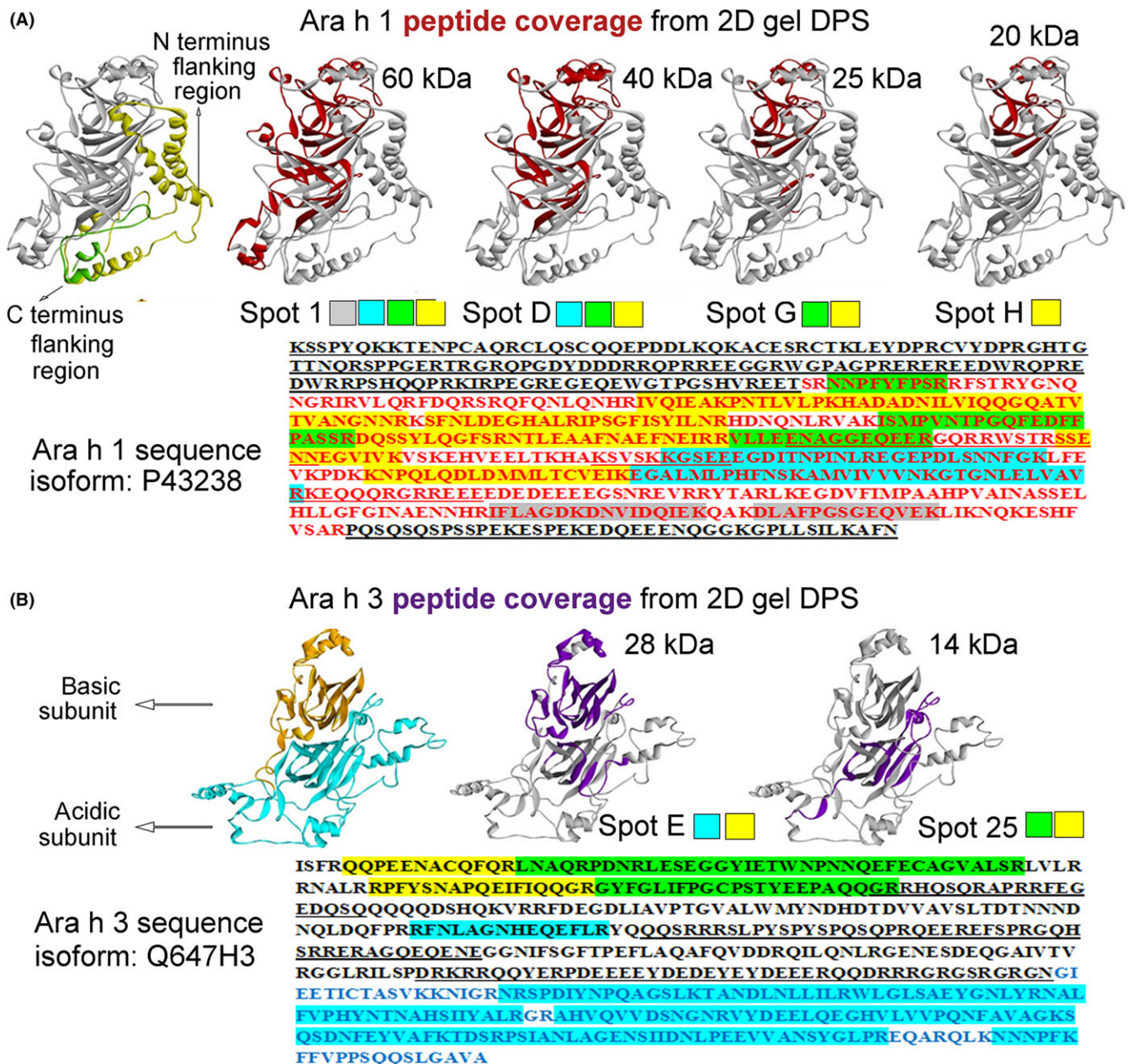


FIGURE 2 Main pepsin digestion-resistant fragments of Ara h 1 and Ara h 3. A, 3D structure of Ara h 1; non-core N-terminal is in yellow, non-core C-terminal is in green, and the core is in grey. The main digestion-resistant peptides of Ara h 1 found in the 2D spots: spot 1 (about 60 kDa), spot D (about 40 kDa), spot G (about 25 kDa) and spot H (about 20 kDa). Peptides are shown in red. B, 3D structure of Ara h 3; the basic subunit is in orange and acidic subunit is in light blue. The main digestion-resistant peptides of Ara h 3 found in the 2D spots: spot E (about 30 kDa) and spot 25 (about 14 kDa). Peptides are shown in violet. The crystal structures of peanut major allergens Ara h 1 (PDB entry 3SMH) and Ara h 3 (PDB entry 3C3V)

3.4 | 2D Immunoblotting of CPS and DPS with the pooled sera of peanut-sensitized patients

Reactivity to CPS and DPS proteins was also demonstrated and compared in immunoblotting experiments with pooled sera of patients sensitized to peanut (Figure 3). The IgE-reactive pattern of pooled sera on CPS and DPS was very similar (Figure 3). In addition, it was noted that Ara h 2 and 6 reactivity was predominant in both CPS and DPS, suggesting that intact Ara h 2 and Ara h 6 are main contributors in IgE reactivity of fraction represented by

large digestion-resistant fragments of the peanut gastric digest. This result supports the findings that Ara h 2 and 6 are major peanut allergens.²⁰

3.5 | IgE-binding potency and protein fold after simulated gastric digestion of peanut were preserved

IgE-binding potency of proteins extracted from CPS and DPS were compared in inhibition ELISA test (Figure 4A) with a pool of sera from ten peanut-sensitized patients (Table S3). As a reference

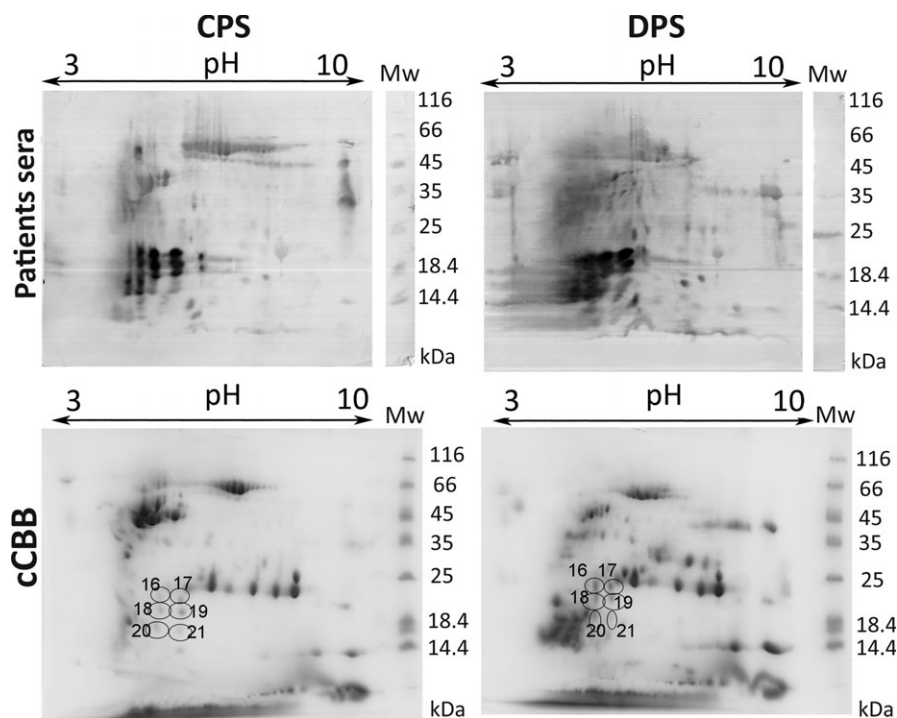


FIGURE 3 Representative 2D immunoblots of control peanut sample (CPS) and digested peanut sample (DPS) probed with the pool of patients sera. Mw, molecular weight markers; kDa, kilodaltons; cCBB, colloidal Coomassie Brilliant Blue dye. From CPS and DPS CBB gels, spots 16-19 (Ara h 2 isoforms found) were excised, together with spots 20-21 (Ara h 6 isoforms determined) and processed for tandem mass spectrometry bottom-up proteomics

material, SPE was used. The IC_{50} values were determined as 1.68, 3.29 and 9.61 $\mu\text{g/mL}$ for the SPE, CPS and DPS, respectively. The narrow range of IC_{50} values (only a threefold difference) between CPS and DPS indicated highly similar IgE-binding potency of these samples.

As IgE-binding potential of the peanut digesta was mostly preserved, the far UV CD spectra of CPS and DPS were assessed to compare secondary structure elements (Figure S3). CD spectrum of CPS showed properly folded forms of proteins, probably from predominant Ara h 1 and Ara h 3. In quantitative sense, in DPS the most of Ara h 1 and Ara h 3 are proteolysed to high molecular mass digestion-resistant fragments (Figure S2), with preserved core of the protein structure. The CD spectrum of DPS showed overlap with spectrum from CPS, explaining preserved protein folded structures. The presence of enzymes during the digestion process caused only negligible change in the secondary structures of peanut proteins, indicating low extent of proteolysis and retention of proper folds of the protein structure.

3.6 | SDRPs contribute to IgE reactivity of the peanut gastric digesta

To obtain additional information on SDRPs (<10 kDa) presence and IgE reactivity, intact proteins and larger digestion fragments were removed by ethanol precipitation and the SDRPs were further purified by gel filtration. We analysed their IgE-binding properties on the solid phase for peanut rAra h 1, rAra h 2 and rAra h 3 (Figure 4B, Figure S4), with individual patients' sera.

Six of the 7 patients reacted to Ara h 1. SDRPs inhibit the binding of IgE to Ara h 1 in sera from 3 of the tested patients (more than

10% of IgE-binding inhibition). In all patients who reacted to Ara h 3, inhibition of IgE binding to Ara h 3 was achieved with the SDRPs of peanut digesta to an extent of 20%-85%. However, the SDRPs gave the highest inhibition of Ara h 2, from 64% to 90% inhibition (Figure 4B, Figure S4), suggesting the presence of potent functional Ara h 2 epitopes in SDRPs fractions. The absence of intact allergens in the tested fractions was confirmed by electrophoresis and immunoblotting using antibodies to Ara h 2/6 (data not shown); peptides were identified by mass spectrometry (Tables S5 and S6).

We searched the IEDB database to match SDRPs released during peanut digestion (Tables S5 and S6) with peanut continuous epitopes recognized for *Homo sapiens* host. With mass spectrometry intact pepsin-generated peptides search, we identified 27 and 18 peptides of Ara h 1 and Ara h 3, with a part of continuous epitope sequences, respectively (Table S5, Figure 5A,B). We also detected 2 non-epitope peptides from Ara h 8 (Table S5). Analysing SDRPs after reduction/alkylation and digestion by trypsin, 2 peptides of Ara h 2, both being part of continuous epitopes, were found (Table S6, Figure 5C), including additional peptides originating from Ara h 1 and Ara h 3 (Table S6, Figure 5A,B). Finally, the digesta profile comprised of 67 peptides of Ara h 3 (30 were a part of continuous epitope sequences) and 31 peptides of Ara h 1 (28 were a part of continuous epitope sequences). Peptides from Ara h 9, Ara h 11 and Ara h 13, originating from the non-epitope regions, were detected. All these SDRPs identified in the ethanol-soluble fraction of the peanut digesta were mostly neglected by low-resolution analytical methods as they were of a size less than 10 kDa. Although there is no literature data providing precise identification of discontinuous epitopes for peanut allergens, identified SDRPs contain many of amino acid residues of discontinuous epitope motifs for Ara h 1,²¹ as well as

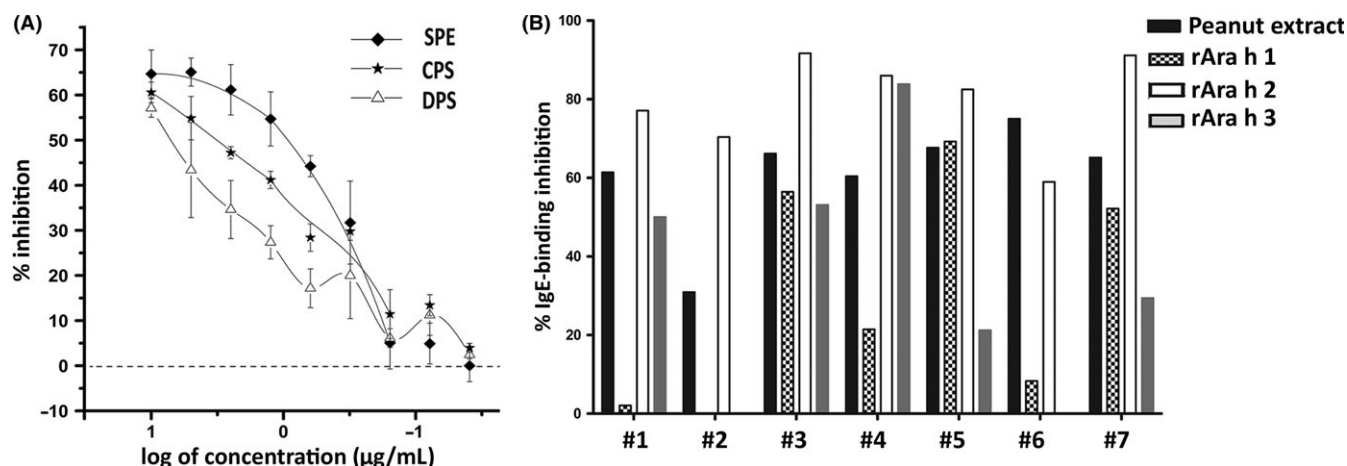


FIGURE 4 IgE-binding properties of control peanut sample (CPS) and digested peanut sample (DPS). A, ELISA inhibition: IgE binding of pooled sera from peanut-sensitized patients to the peanut extract (SPE) coupled to the plate was inhibited by SPE itself and liquid fraction of CPS and DPS; B, ImmunoCAP inhibition of specific IgE on solid phase by short digestion-resistant peptides (SDRPs) obtained during peanut digestion. On x-axis numbers denote patients in Table S3

residues which are part of consensus amino acid pattern of Ara h 2 and Ara h 6 discontinuous epitopes²² (Figure S5).

3.7 | SDRPs of peanut allergens were mostly hydrophobic

Hydrophobicity index was analysed for the isolated SDRPs. Data showed that SDRPs of Ara h 1, Ara h 2, Ara h 3 and Ara h 6 were markedly more hydrophobic than rest of the sequences (Figure S5), suggesting that they have strong propensity to aggregate via non-covalent hydrophobic interactions. Also, most of SDRPs on solvent accessible surface are part of nonpolar and hydrophobic area on protein surface rather than polar/charged 1 (Figures S7, S8 and S9). It has previously been shown that Ara h 1 small peptides obtained after *in vitro* gastric digestion retained their sensitizing and the reacting potential, due to aggregation of peptides induced by hydrophobic interactions.¹¹ Moreover, polar regions of peanut allergens were not present in the identified SDRPs released by pepsin digestion, suggesting their preferential cleavage by proteases.

4 | DISCUSSION

In this study, we identified the structures and assessed IgE reactivity of whole peanut grain pepsin-resistant allergens, their larger fragments and SDRPs released by pepsin digestion of whole peanut grain under *in vitro* static digestion protocol mimicking physiologically relevant conditions.⁵ We demonstrated the presence of intact Ara h 2 and Ara h 6, including small portion of intact Ara h 1 and 3, large digestion-resistant fragments of Ara h 1 and Ara h 3, and mixture of SDRPs mainly comprised of Ara h 1, Ara h 2, Ara h 3, and in lesser extent of Ara h 8, Ara h 9, Ara h 11 and Ara h 13, in the pepsin digesta. In addition, we have shown that following pepsin digestion, reactivity of IgE to 2S albumins predominates in digesta due to

preservation of intact protein, but also due to the presence of potent IgE epitopes in SDRPs.

Identification of peptides resistant to *in vitro* digestion is an important factor in the assessment of food proteins allergenicity, as immunologically active digestion-resistant peptides in the intestinal lumen can trigger immune responses in susceptible individuals. In earlier studies, peanut allergens were detected in breast milk, suggesting that digestion-resistant immunoreactive fragments of peanut allergens can reach the circulation.²³ Moreover, a recent study showed the presence of IgE-reactive peptides after gastric/intestinal and brush border proteases digestion of whole peanut grains.⁹

In vitro digestion studies with purified proteins reported dramatically higher protein digestibility than possible under physiological conditions. In addition, it is well known that pure proteins in solution can have different sensitivities to proteolysis compared to proteins adsorbed at oil-in-water interface owing to changes in the protein structure.^{18,24} Therefore, the proteins regarded as highly digestible, as estimated by pepsin digestion in their purified form, could be markedly resistant to proteolysis within a complex food matrix.

Ara h 1 and Ara h 3 acidic forms were shown to be susceptible to pepsin digestion which is in accordance with Vieths et al.⁶ 2D SDS-PAGE coupled with MS/MS has shown pepsin-resistant Ara h 1 forms in spots D, G, H and M, corresponding to the forms found in the study of Koppelman et al, after pepsin digestion of purified Ara h 1 for 0.25 minutes.⁷ Similarly, pepsin-resistant Ara h 3 forms found in spots 9-15, 23-25, N, M and O correspond to forms found in the same study at the mentioned conditions with the purified Ara h 3.⁷ Therefore, in the presence of the food matrix, similar digestion-resistant forms of Ara h 1 and 3 were demonstrated to be stable for 120 minutes under the gastric digestion conditions, providing a rough estimate of Ara h 1 and Ara h 3 being 500 times less digestible by pepsin in the whole peanut grain than in solution.

Di Stasio et al⁹ reported that after gastrointestinal digestion, Ara h 1 was almost completely digested and no peptides larger than

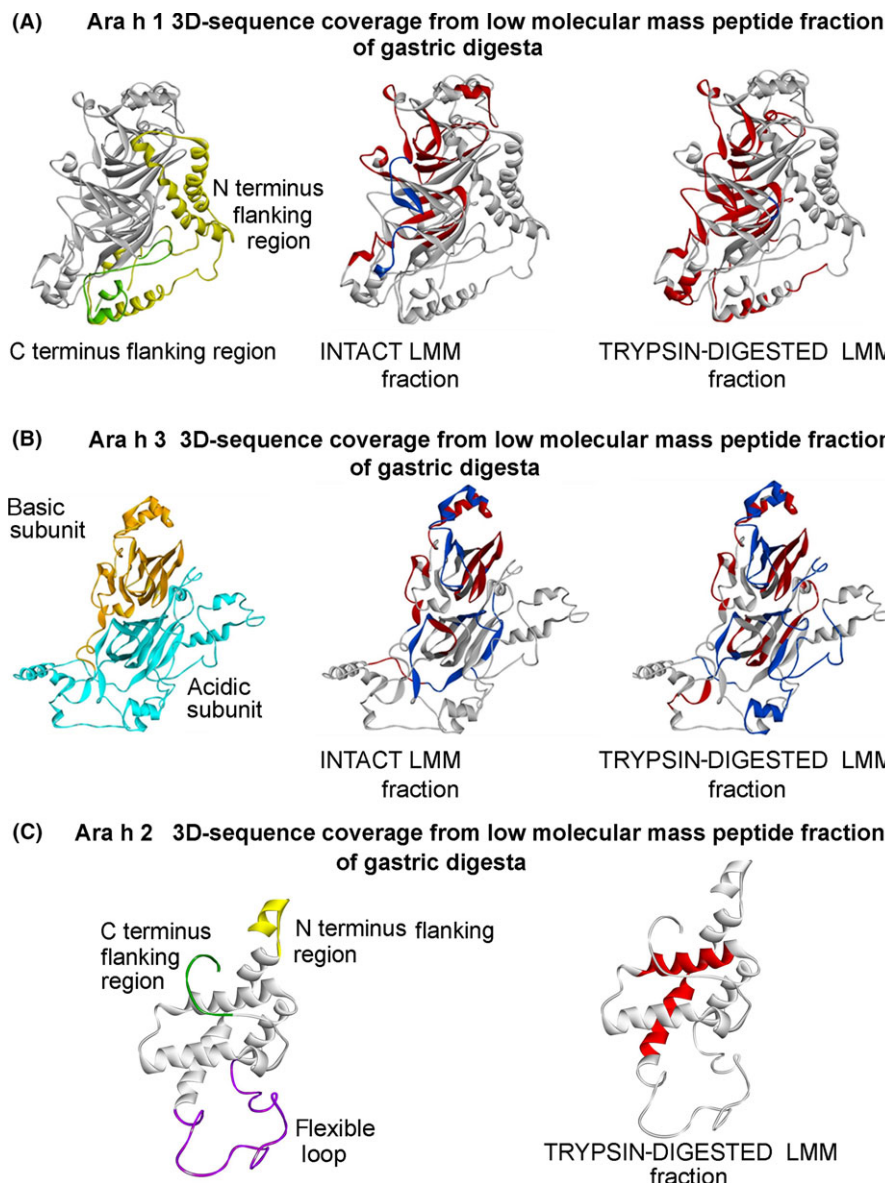


FIGURE 5 Regions with identified peptides of Ara h 1, Ara h 2 and Ara h 3 found in the short digestion-resistant peptides (SDRPs) of peanut digested by pepsin. A, 3D structure of Ara h 1; non-core N-terminal is in yellow, non-core C-terminal is in green, and the core is in grey. Intact peptides (middle) and peptides found after reduction, alkylation and trypsin digestion of SDRPs of peanut digested by pepsin (right). Regions with peptides matched with peanut continuous epitopes (IEDB database search) are in red, and non-matching peptides are in blue. B, 3D structure of Ara h 3; the basic subunit is in orange, and acidic subunit is in light blue. Intact peptides (middle) and peptides found after reduction, alkylation and trypsin digestion of short digestion-resistant peptides (SDRPs) of peanut digested by pepsin (right). Regions with peptides matched with peanut continuous epitopes (IEDB database search) are in red, and non-matching peptides are in blue. The crystal structures of peanut major allergen Ara h 1 (PDB entry 3SMH), Ara h 2 (PDB entry 3OB4) and Ara h 3 (PDB entry 3C3V). C, 3D structure of Ara h 2; flexible loop is shown in violet colour, C-terminus flanking region is shown in green colour, and N terminus flanking region is shown in yellow colour. Peptides found after reduction, alkylation and trypsin digestion of SDRPs of peanut digested by pepsin are shown in red colour, and they are matched with peanut continuous epitopes (IEDB database search)

6 kDa were detected. The most of Ara h 1 was proteolysed to a digestion-resistant fragment of 60 kDa, and half of it was further digested, resulting in cascade pattern of Ara h 1 pepsin proteolysis (Figure 2A). Thus, half of initial quantities of 60 kDa digestion-resistant core of Ara h 1, before proteolysis in the intestinal phase, could pass into intestinal mucosa where conserved epitopes could trigger immunoreactions.

In contrast to the acidic subunits, the basic Ara h 3 subunit was more resistant to pepsin digestion. Acidic subunits of Ara h 3 originated from the N-terminal domain, while basic subunits originated from the C-terminal domain. N-terminal domain contained 3 long regions of disordered structure (G119-Q138, Q212-G259 and D311-N345, Figure 2B), occupying one-third of N-terminal domain, while C-terminal domain contained only 1 short disordered region at the

end of the sequence (S522-A530, Figure 2B), occupying <2% of the C-terminal domain.²⁵ This explains why the acidic subunits are much more prone to pepsin proteolysis than the more compact basic subunit and also why natural Ara h 3 processing results in higher diversity in the mass of acidic subunits (13-45 kDa), as compared to the basic subunits (about 23 kDa).

Almost completely preserved cores of these 2 allergens explain the similarity in the secondary structures of digested and undigested samples observed by CD spectrometry.

In IgE ELISA inhibition, the same order of magnitude of IC₅₀ values obtained for control and digested sample suggests that partly digested peanut allergens mainly retained their allergenic potential. Pepsin proteolysis only slightly reduced the IgE-binding potential of peanut proteins extracted during digestion. These results are in agreement with data shown in 2D immunoblots.

We also examined the IgE reactivity of SDRPs (<10 kDa) from the gastric-simulated digesta by an inhibition study on peanut allergens on ImmunoCAP and demonstrated that 2S albumin SDRPs are more potent than Ara h 1 and Ara h 3 SDRPs. This peptide fraction could be the result of extensive proteolysis of peanut proteins, thus representing the digestion-resistant peptides of the smallest molecular mass, or they could be truncated parts of larger DRPs, excised following proteolytic attack on exposed loops of peanut's proteins.

Although Ara h 1 and Ara h 3 are the most abundant storage proteins in peanuts, patients with peanut allergies recognize Ara h 2 and Ara h 6 more frequently and with greater intensity.²⁰ IgE binding to Ara h 2 and Ara h 6 is primarily dependent on the discontinuous epitopes,^{26,27} and it is likely that CMCEALQQIMENQ peptide that we have identified in SDRP fraction, besides being a part of continuous Ara h 2 and Ara h 6 epitopes, could be a part of a potent discontinuous epitope by creating S-S crosslinking adducts.

Schocker et al²⁷ investigated the transfer of Ara h 2 in human breast milk and identified 5 tryptic fragments of Ara h 2, of which 2 (ANLRPCEQHLMQK and CMCEALQQIMENQSDR) were same as the peptides that we identified in this study in SDRP fraction of gastric digesta. These peptides could be part of the same discontinuous epitope held together by several disulphide bridges.

The results of our study demonstrated that after gastric digestion of whole peanut matrix, high molecular mass digestion-resistant fragments of Ara h 1, 2, 3 and 6, with mostly retained core structure bearing discontinuous epitopes, survive. On the other hand, large proportion of generated SDRPs are part of continuous epitopes resulting in high allergenic potential of SDRPs. Therefore, it can be expected that in vivo these species become exposed to the intestinal immune system and transported to circulation. Increased intestinal permeability (as found under different physiological and pathological conditions) or disruption of tight junctions may enable transport of peanut protein fragments across the intestinal epithelium. Therefore, in peanut allergic individuals, in both intestine and circulation, they can induce allergic reaction. In intestine, these fragments can induce intestinal anaphylaxis via mast cell-dependent IgE-FcεRI-IL-13 pathway²⁸ and histamine-dependent mesenteric lymph node and lamina propria DC accumulation.²⁹

In conclusion, we demonstrated that the most potent allergens, Ara h 2 and Ara h 6, remained mostly intact to proteolysis by pepsin, and SDRPs originating from Ara h 2 were most potent in inhibiting IgE binding, suggesting that not only intact Ara h 2 but also its SDRPs are of clinical relevance. Ara h 1 N- and C-terminal parts and acidic forms of Ara h 3 were most susceptible to proteolysis by pepsin. However, Ara h 1 exhibited sequential digestion into a series of DRPs with preserved allergenic capacity. Thus, the major peanut allergens and SDRPs play important roles in allergic reactions to peanut.

Compared to the findings of in vitro digestion studies performed on purified proteins under similar conditions, digestibility of proteins when they are within real food matrix was dramatically lower.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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